REAL-TIME FLUOROGENIC POLYMERASE CHAIN REACTION (PCR) ASSAYS TO NUCLEIC ACID-BASED DETECTION OF SIMULANTS AND BIOTHREAT AGENTS

Akbar S. Khan, Kevin P. O'Connell, Jennifer R. Bucher, Patricia E. Anderson, Cheng J. Cao, Mark V. Gostomski, and James J. Valdes
U. S. Army Soldier and Biological Chemical Command, Edgewood Chemical Biological Center
AMSSB-RRT-BM, Aberdeen Proving Ground, MD 21010

ABSTRACT

Polymerase chain reaction (PCR) is an exquisitely sensitive method for the amplification and detection of genetic material (DNA and RNA sequences). We have developed several assays using a variation of PCR that proceeds very quickly and allows the monitoring of the progress of the reaction in real time. We describe here assays for the detection of the gene encoding staphylococcal enterotoxin A (SEA), a toxin produced by the bacterium *Staphylococcus aureus* and an agent responsible for a significant fraction of food poisoning incidents worldwide. We also describe several assays for the detection of a simulant of viral pathogens, the bacteriophage MS2.

INTRODUCTION

The use of antibodies, proteins that bind their targets with great specificity and strong affinity, has long been a cornerstone technology for the detection of biological warfare agents. Immunological assays are currently part of biodefense systems fielded by the US armed forces. The role of immunological detection of BW agents will continue to be integral. However, new technologies have emerged in the last several years that are similarly specific and sensitive and can provide orthogonal confirmation of immunological results, or results at all when immunological methods are not sufficiently sensitive. Advances in nucleic acid biochemistry, namely the polymerase chain reaction (PCR) and associated fluorescent chemistries, have made available new methods for detecting the presence of genes from BW agents with exquisite sensitivity.

Traditional PCR uses a thermostable DNA polymerase in a DNA synthesis reaction, primed by DNA oligonucleotides that are complementary to a specific sequence within the target DNA. Standard PCR (in the absence of probe DNA) results in a doubling of the number of copies of target sequence after each round of DNA synthesis, and a geometric increase in number of copies after each reaction cycle. The product can be observed afterwards by separation of the DNA by agarose geelectrophoresis.

Real-time fluorescent PCR works similarly, with the addition of a third small fragment of DNA to the reaction mixture. The DNA/RNA detection reaction combines standard PCR with a third reagent, a probe DNA molecule that hybridizes to a target sequence between the sequences bound by the two PCR primers. The probe is labeled at one end with a fluorescent dye molecule and at the other end with a molecule that quenches the fluorescence of the dye molecule, such that the proximity of these two

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01 JUL 2003	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Real-Time Fluorogenic Polymerase Chain Reaction (Pcr) Assays To Nucleic Acid-Based Detection Of Simulants And Biothreat Agents	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **U. S. Army Soldier and Biological Chemical Command, Edgewood Chemical Biological Center AMSSB-RRT-BM, Aberdeen Proving Ground, MD 21010**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES
See also ADM001523.

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**UU**	**7**	

molecules results in a quenching of the dye's fluorescence. When a thermostable DNA polymerase extends one of the two primers into the area where the probe is bound, the 5' nuclease activity of Taq DNA polymerase degrades the probe and releases the fluorescent and quencher molecules bound to the probe ends. The separation of the dye and the quencher results in an increase in the overall fluorescence of the sample mixture. A detector in the PCR instrument continually monitors and records the fluorescence present in the sample. Significant accumulation of fluorescence in the sample above background level indicates a positive detection of the target DNA.

With the explosion of knowledge in the sequences of entire microbial genomes comes a rich "target space" from which to obtain sequences that are specific for a particular strain of a pathogen, or a target shared by many strains and species of a pathogen. We have used this knowledge to develop a large number of assays for the detection of genes from several pathogens and toxin-producing organisms. This paper describes the use of real-time fluorogenic PCR for the specific detection of nucleic acids from a toxin-producing bacterium, *Staphylococcus aureus*, and a simulant for viral pathogens, bacteriophage MS2. The SEA assay can also be useful in the detection of toxigenic *S. aureus* in food samples, or for the detection of *S. aureus* DNA in partially purified samples of SEA itself. Assays for MS2 are useful in the development of new PCR platforms, where investigators do not have access to DNA/RNA from viral pathogens (as is increasingly the case). MS2 is also used in field trials for evaluating BW agent detectors, so assays for MS2 are also necessary for evaluating the usefulness of PCR platforms in testing situations.

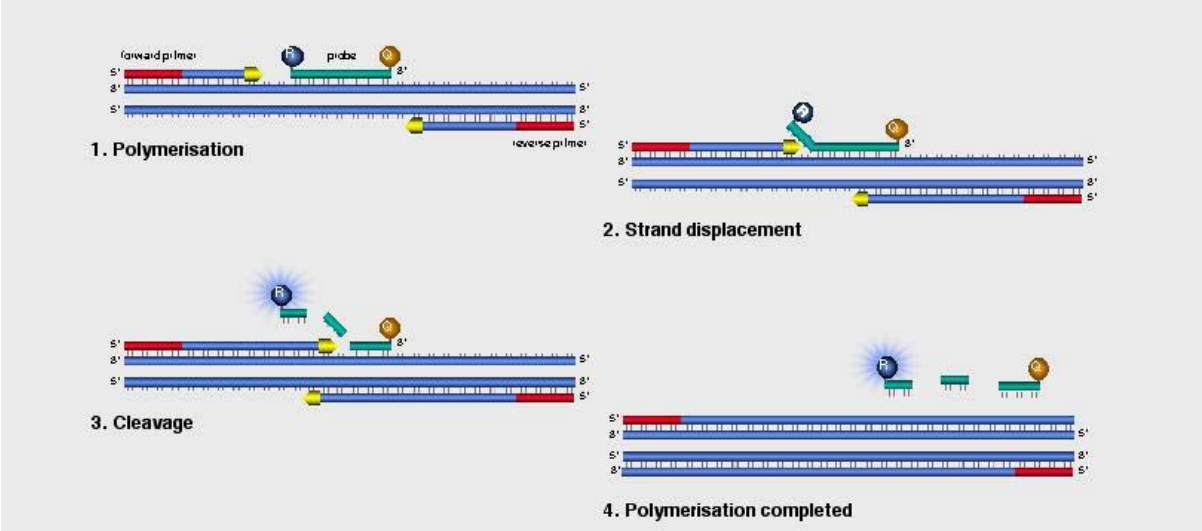

Figure 1. Schematic of real-time fluorogenic ("TaqMan®") polymerase chain reaction (PCR).

In Figure 1, one cycle is shown. As Taq DNA polymerase synthesizes the new top strand of DNA (upper left), it cleaves the probe DNA that is in its path, releasing the fluorescent dye molecule from interacting with the quencher (bottom right).

METHODS

Viral and bacterial strains, growth conditions, and DNA purification

Bacteriophage MS2 (ATCC 15597-B1), its *Escherichia coli* host strain (ATCC 15597), and several strains of *Staphylococcus aureus* (ATCC51651 and ATCC13565) were obtained from the American Type Culture Collection (Manassas, VA). *E. coli* and *S. aureus* were grown in Luria-Bertani (LB) medium. Genomic RNA from MS2 virions was isolated with TRIzol reagent (Invitrogen, Inc.) according to the

manufacturer's instructions. Genomic DNA from strains of *S. aureus* was isolated with the DNeasy Tissue Kit (QIAGEN, Inc., Valencia, CA).

Assay design

Assays for MS2 detection were designed using the whole genomic sequence of MS2 (GenBank Accession # NC_001417). Assays for *entA* (the gene encoding SEA) were designed using bases 282 to 1035, the open reading frame encoding SEA from GenBank accession L22566. Sequence data were analyzed with the Lasergene software package (DNASTAR, Madison, WI). Primers and probes were designed using Primer Express software (Applied Biosystems, Foster City, CA). All assays designed adhered to the software's defaults for probe and primer characteristics, including primer and probe length, primer and probe T_m, T_m differential between primers and probe, C/G ratio in probe sequence, and when possible, no runs of a single base longer than four and at least three of the last five primer bases (3' end) are A or T. Candidate amplicon sequences were searched for homology with known sequence data (GenBank) using NCBI-BLAST.

PCR conditions: preliminary assays

Primer pairs were ordered initially without probe to test their ability to amplify target DNA or RNA sequences. Preliminary assays were performed using the reagents in Applied Biosystems EZ PCR (or RT-PCR, for MS2) reagent sets in accordance with the manufacture's instructions. DNA assays were assembled as follows: 25 µl PCR master mix, 4 µl forward primer (5 µM), 4 µl reverse primer (5 µM), 1 µl target template DNA (1 ng total DNA), 16 µl H_2O. Two aliquots of this mixture were placed into wells of a 384-well assay plate. The plate was placed in the sample tray of an Applied Biosystems ABI PRISM™ 7900 Sequence Detection System, programmed as follows: 1 cycle of 50°C, 2 min; 1 cycle of 95 °C, 10 min; 40 cycles of 95 °C, 15 sec and 60 °C, 1 min.

RNA assays were assembled as follows: 25 µl 2X Master Mix, 1.25µl 40X MultiScribe and Rnase Inhibitor Mix, 4µl forward primer (5 µM), 4 µl reverse primers (5 µM), 1 µl target template RNA (1 ng total RNA), 14.75 µl H_2O. Two aliquots of this mixture were placed into wells of a 384-well assay plate. The plate was placed in the sample tray of an Applied Biosystems ABI PRISM™ 7900 Sequence Detections System, programmed as follows: 1 cycle of 48°C, 30 min; 1 cycle of 95°C, 10 min; 45 cycles of 95°C, 15 sec and 64°C, 1 min.

The contents of each well were removed and analyzed by gel electrophoresis. Each candidate primer pair was tested twice; probe for an assay was ordered only when the corresponding primer pair yielded amplified DNA detectable by gel electrophoresis. Probe DNA was obtained from Applied Biosystems (Foster City, CA). Assays with probe were assembled in a similar fashion, but with the inclusion of probe DNA. One nanogram of target DNA or of target RNA template was added to reactions; one ng of non-target DNA/RNA was added to tests for cross-reactivity.

RESULTS

Assays for the detection of the viral simulant bacteriophage MS2

We developed five separate assays for MS2, which detect sequences in each of the four genes encoded by the phage genome (assembly protein, coat protein, lysis protein, and RNA replicase). Representative data for the assays are shown in Figure 2. The limit of detection (LOD) for MS2 genomic RNA in this assay was less than 10 femtograms of purified genomic RNA (Ct = ~33). The other four assays had LODs between 1 fg and 100 fg (data not shown).

Data for only one of the four MS2 assays is shown for the sake of brevity. Similar assays were also performed with the MS2 assay primer sets and reaction mixtures including the dye SYBR® Green, which selectively fluoresces when binding double-stranded DNA (ds-DNA) (data not shown). This assay format relieves the requirement for a specific probe molecule and in many cases is even more sensitive.

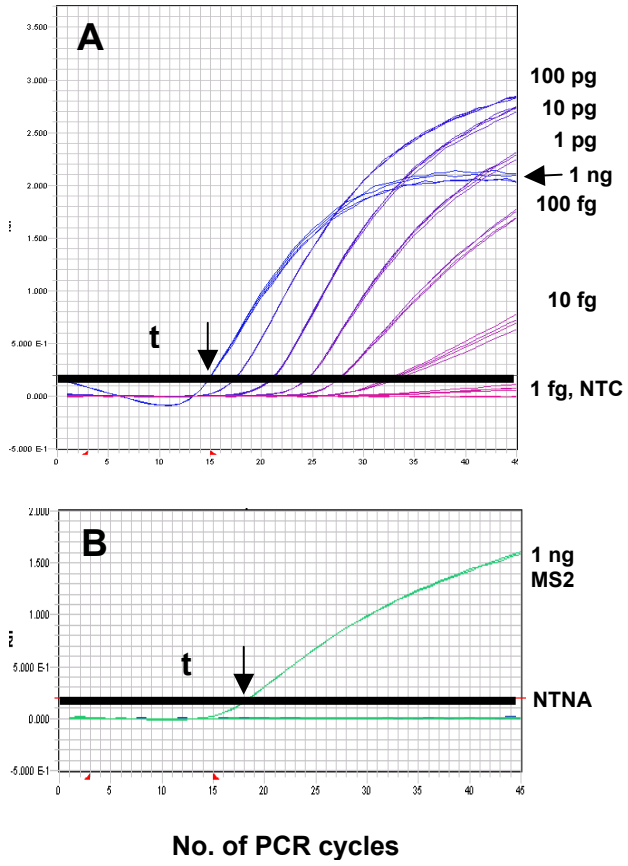

Figure 2. Detection of bacteriophage MS2 genomic RNA by real-time fluorogenic RT-PCR.

Thick horizontal lines represent the threshold (t) for significant signal detection. Larger amounts of RNA permitted positive detection (signal above threshold) with fewer PCR reaction cycles. The greatest amount of RNA (1 ng) produced a signal with the fewest number of cycles (arrows). **A**, limit of detection experiments demonstrating the sensitivity of assay MS2-1, performed using ten-fold dilutions of a preparation of bacteriophage MS2 RNA (concentrations at right). Using the probe and primer set in assay MS-1, 10 fg of RNA were repeatably and reproducibly detected. Assay MS-1 did not detect 1 fg of MS2 RNA (assay gave results indistinguishable from the negative (no-template) control, NTC). **B,** cross-reactivity experiments using primers and probe from assay MS2-1. None of the non-target nucleic acid (NTNA) samples were amplified above threshold by the MS2-1 primer set, while 1 ng of MS2 RNA gave a positive reaction.

All five assays for MS2 were tested against a panel of DNA or RNA from non-target organisms to determine whether genomic DNA from other organisms might inadvertently give a positive signal (including human DNA, which might contaminate a sample when introduced from an operator). Tested at a relatively high amount (1 ng DNA or RNA per assay tube), none of the non-target nucleic acids

produced a signal in any of the assays we developed (Figure 2b; the non-target nucleic acids are listed in Table 1, below). Assays MS2-2 through MS2-5 gave similar results (data not shown).

Assays for the detection of the *entA* gene, encoding staphylococcal enterotoxin A (SEA)

Two assays for the *entA* gene were developed and tested in a fashion similar to that used to develop the MS2 assays. Representative data for the assays are shown in Figure 3. The assays were developed using purified genomic DNA from *S. aureus* strain ATCC 13565. The limit of detection for *S. aureus* genomic DNA in these assays was approximately 100 femtograms (Ct = ~37). The assays also gave positive results when tested against genomic DNA from ATCC51651, which was not listed by ATCC as producing SEA (data not shown). We have a collection of *S. aureus* strains obtained from clinical samples and are working to correlate SEA production (as detected by immunoassay) and the presence of the *entA* gene.

Similar assays are currently being performed with the SEA assay primer sets and reaction mixtures including the dye SYBR® Green, which selectively fluoresces when binding double-stranded DNA (ds-DNA) (data not shown). This assay format relieves the requirement for a specific probe molecule and in many cases is even more sensitive.

Both assays for SEA were also tested against a panel of DNA from non-target organisms to determine whether genomic DNA from other organisms might inadvertently give a positive signal (including human DNA, which might contaminate a sample when introduced from an operator). Tested at a relatively high amount (1 ng DNA or RNA per assay tube), none of the non-target nucleic acids produced a signal in any of the assays we developed (Figure 3c; the non-target nucleic acids are listed in Table 1).

Table 1. Results of cross-reactivity experiments for two assays that detect the *entA* gene

Primer/Probe	**177/178/AP3**		**179/180/AP4**	
	Experiment #			
Template DNA	1	2	1	2
Bacillus anthracis pX01	-	-	-	-
Bacillus anthracis pX02	-	-	-	-
Homo sapiens	-	-	-	-
Clostridium perfringens	-	-	-	-
Yersinia pestis EV-76	-	-	-	-
Francisella tularensis Schu 4	-	-	-	-
Salmonella typhimurium LT2	-	-	-	
Ricinus communis L.	-	-	-	-
S. aureus ATCC13565 (EntA)	+	+	+	+

All of the "+" or "-" represent 4 replicate assays.
SEA DNA: 10 pg/assay; All of other DNA: 1 ng/assay;

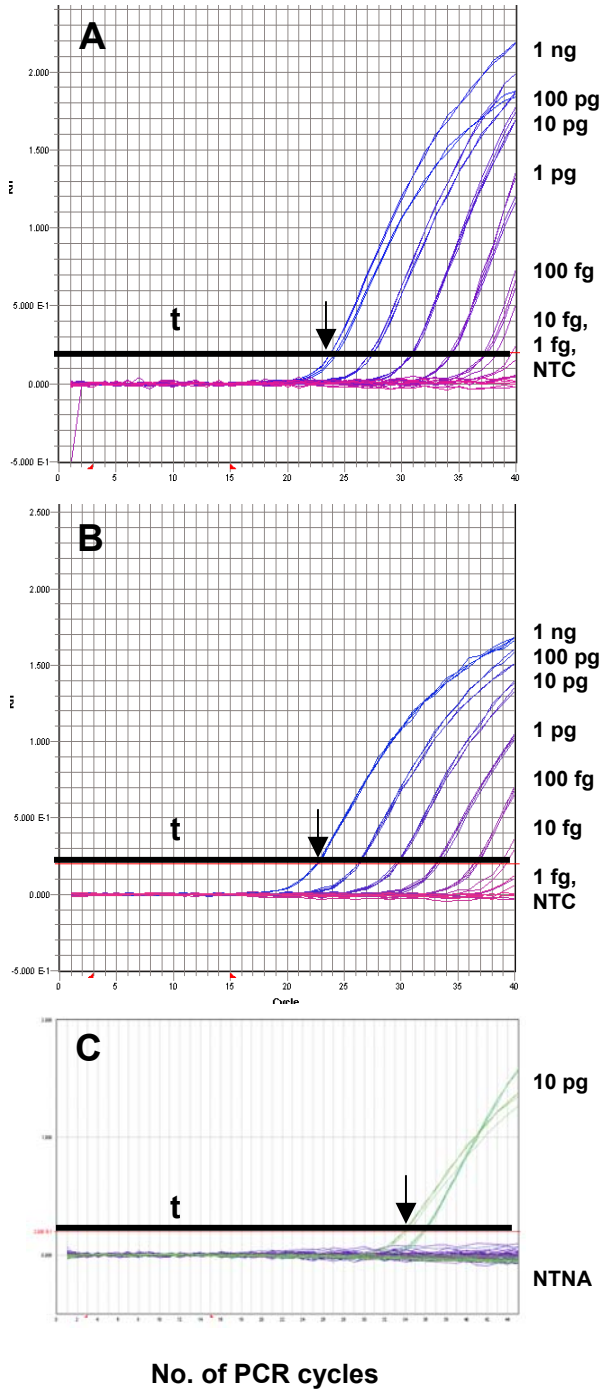

Figure 3. Detection of *S. aureus* genomic DNA by real-time fluorogenic PCR.

Thick horizontal lines represent the threshold (t) for significant signal detection. Larger amounts of DNA permitted positive detection (signal above threshold) with fewer PCR reaction cycles. The greatest amount of DNA (1 ng) produced a signal with the fewest number of cycles (arrows). **A**, limit of detection experiments demonstrating the sensitivity of assay SEA-1, performed using ten-fold dilutions of a preparation of *S. aureus* ATCC13565 DNA (concentrations at right). Using the probe and primer set in assay SEA-1, 100 fg of DNA were repeatably and reproducibly detected. Assay SEA-1 did not detect 10 fg or 1 fg of *S. aureus* DNA (assay gave results indistinguishable from the negative [no-template] control, NTC). **B**, limit of detection experiments demonstrating the sensitivity of assay SEA-2,

performed as described above. Using the probe and primer set in assay SEA-2, 100 fg of DNA were repeatably and reproducibly detected. Assay SEA-2 also did not detect 10 fg or 1 fg of *S. aureus* ATCC13565 DNA. **C,** cross-reactivity experiments using primers and probe from assays SEA-1 and SEA-2. None of the non-target nucleic acid (NTNA) samples (listed in Table 1) were amplified above threshold by the SEA-1 and SEA-2 primer sets, while 10 pg of *S. aureus* ATCC13565 DNA gave a positive reaction.

DISCUSSION

Primers 177, 178, and probe AP3 constitute one assay, and primers 179, 180, and probe AP4 constitute the second assay. The actual fluorograms are shown in Figure 3c. All of the assays described here are based on the 5' nuclease assay, which exploits the 5' nuclease activity of Taq polymerase to cleave a non-extendable, dual-labeled fluorogenic probe as described above (Olive *et al.* 1999, Holland *et al.* 1991). A number of workers, including those in other U.S. Army laboratories, are developing assays using this technology against a broad spectrum of pathogens and other threat agents. The chemistry of these assays should be amenable to lyophilization and storage in a stable, dry form with a long shelf life and minimal required infrastructure. However, like all PCR assays, the determination of a positive result can be obtained by direct observation of the PCR products in an agarose gel, or through the use of intercalating dyes (such as SYBR Green) that do not require the 5' to 3' exonuclease activity of a thermostable polymerase. There are currently on the market, or under development, several platforms, from lab scale (such as our ABI 7900) to "field-ruggedized" instruments (such as the R.A.P.I.D. from Idaho Technologies) to hand-held equipment (for example, the BioSeeq^{tm} from Smiths-Environmental Technologies Group, Inc.) that are designed to run assays using "TaqMan"-style chemistries. This gives US Armed forces field units, domestic preparedness responders and public health workers a spectrum of biological detection and identification capabilities that can be used in a variety of settings.

REFERENCES

1. Olive, D. M., and Bean, P. 1999. Principles and applications of methods for DNA-based typing of microbial organisms. J. Clin. Microbiol. 37: 1661-1669.

2. Holland, P. M., Abramson, R. D., Watson, R. and Gelfand, D. H. 1991. Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of *Thermus aquaticus*. Proc. Natl. Acad. Sci. USA 88:7276-7280.